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Isolation and Characterization of Mutants Defective in the Cyanide-Insensitive Respiratory Pathway of *Pseudomonas aeruginosa*

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The branched respiratory chain of *Pseudomonas aeruginosa* contains at least two terminal oxidases which are active under normal physiological conditions. One of these, cytochrome *co*, is a cytochrome *c* oxidase which is completely inhibited by concentrations of the respiratory inhibitor potassium cyanide as low as 100 μM. The second oxidase, the cyanide-insensitive oxidase, is resistant to cyanide concentrations in excess of 1 mM as well as to sodium azide. In this work, we describe the isolation and characterization of a mutant of *P. aeruginosa* defective in cyanide-insensitive respiration. This insertion mutant was isolated with mini-D171 (a replication-defective derivative of the *P. aeruginosa* phage D3112) as a mutagen and by screening the resulting tetracycline-resistant transductants for the loss of ability to grow in the presence of 1 mM sodium azide. Polarographic studies on the NADH-mediated respiration rate of the mutant indicated an approximate 50% loss of activity, and titration of this activity against increasing cyanide concentrations gave a monophasic curve clearly showing the complete loss of cyanide-insensitive respiration. The mutated gene for a mutant affected in the cyanide-insensitive, oxidase-terminated respiratory pathway has been designated *cio*. We have complemented the azide-sensitive phenotype of this mutant with a wild-type copy of the gene by in vivo cloning with another mini-D element, mini-D386, carried on plasmid pADD386. The complemented *cio* mutant regained the ability to grow on medium containing 1 mM azide, titration of its NADH oxidase activity with cyanide gave a biphasic curve similar to that of the wild-type organism, and the respiration rate returned to normal levels. Spectral analysis of the cytochrome contents of the membranes of the wild type, the *cio* mutant, and the complemented mutant suggests that the *cio* mutant is not defective in any membrane-bound cytochromes and that the complementing gene does not encode a heme protein.

Pseudomonas aeruginosa preferentially obtains its energy via aerobic respiration, but although it is often thought of as a strict aerobe, it is well adapted to conditions of limited oxygen supply (28). It is capable of anaerobic growth with nitrate as the terminal electron acceptor (9, 42, 44), and in its absence it is able to ferment arginine, generating ATP by substrate-level phosphorylation (32, 41).

P. aeruginosa, in common with most bacteria, has a branched aerobic respiratory chain composed of substrate-specific dehydrogenases, ubiquinone, and cytochromes (22–25, 44). If NADH-dependent O_2 uptake in the cytoplasmic membranes from this bacterium is measured as a function of cyanide concentration (a classical inhibitor of cytochrome oxidases [18]), then a biphasic titration curve is obtained, indicating the presence of at least two O_2-reactive terminal oxidases (25). One of these is a cytochrome *c* oxidase called cytochrome *co*, which is sensitive to micromolar concentrations of cyanide (50% inhibitory concentration [IC_{50}] = 5 μM cyanide). A second branch of the respiratory chain is terminated by a highly cyanide-resistant oxidase which is inhibited only by millimolar concentrations of cyanide (IC_{50} = 30 mM [25]). This oxidase is also resistant to antimycin A, a specific inhibitor of ubiquinol: cytochrome *c* oxidoreductase complexes and salicylhydroxamic acid, which inhibits the cyanide-resistant, alternative terminal oxidases of plant and fungal mitochondria (4, 19). The cyanide-insensitive oxidase (CIO) of *P. aeruginosa* has not been extensively studied, but it has been observed that the appearance of cyanide-insensitive respiration during the onset of the stationary phase of growth is not accompanied by qualitative or quantitative changes in the cytochrome composition of the respiratory chain (39, 40). This has led to the suggestion that CIO may be a non-heme oxidase quite distinct from anything found in eukaryotic mitochondria or the terminal oxidases of other bacterial respiratory chains (25, 44). However, at present, there is little or no hard evidence to support this idea.

Recently, a third terminal oxidase, a cytochrome baa_3, has been purified from a strain of *P. aeruginosa*. It is sensitive to cyanide (IC_{50} = ~50 μM [9a]), and it is probably a quinol oxidase in vivo (10). However, this oxidase has not been observed in all strains of *P. aeruginosa* (23).

A large body of evidence on the structures and genetic organizations of bacterial terminal oxidases, in particular those related to the mitochondrial cytochrome *c* oxidases, has accumulated. Genes encoding subunits of aa_3-type cytochrome *c* oxidases have been cloned from a number of bacteria, including *Paracoccus denitrificans*, *Bacillus subtilis*, *Bradyrhizobium japonicum*, and *Rhodobacter sphaeroides* (11, 13, 36, 38). It has become clear from this work that aa_3-type cytochrome *c* oxidases constitute a superfamily of respiratory oxidases (5, 12, 13, 35) which also includes aa_3-type quinol oxidases and *o*-type quinol oxidases, such as the cytochrome *bo* of *Escherichia coli* (7). Not all bacterial oxidases belong to this superfamily. In contrast, the cytochrome *bd* quinol oxidases of *E. coli* and *Azotobacter vinelandii* are distinct enzymes with no homology to aa_3-type oxidases (15, 26). Cytochrome *bd* is responsible for

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TABLE 1. *P. aeruginosa* strains

Strain	Description or genotype	Source or reference
PAO6049	*met-9011 amiE200 strA*	D. Haas
PAO7701	PAO6049 *cio*	This work
PAO7702	PAO7701(pLC1), Cio^+	This work
PAO7703	PAO7701(pLC2), Cio^+	This work
PAO7704	PAO7701(pLC3), Cio^-	This work
PAO4141	*met-9020 pro-9024 blaP9202 blaJ9111 aph-9001* FP	8
CD10	PAO4141::D3112cts	8

cyanide-insensitive respiration in *E. coli*, and indeed a survey of the cytochrome composition of bacteria which can perform cyanide-insensitive respiration (defined as respiration in the presence of 1 mM KCN) found that many of these bacteria contain spectrophotometrically detectable cytochrome *bd* (1). However, a significant number of these bacteria, including *P. aeruginosa*, do not.

Cyanide insensitivity may be an incidental property of the *P. aeruginosa* CIO and may be of little physiological importance. However, the ability of *P. aeruginosa* cultured under certain low-O_2 conditions (6) to synthesize hydrogen cyanide as a metabolic product at concentrations that would completely inhibit the cytochrome *co* oxidase suggests that CIO might have a role to play in allowing aerobic respiration under cyanogenic growth conditions and so may be important in allowing *P. aeruginosa* to grow and compete successfully with other bacteria in mixed populations. Cyanide concentrations rise to 200 to 300 μM in *P. aeruginosa* cultures (16a), and interestingly, cyanide can be detected at sites of *P. aeruginosa* infection, including burn eschar, liver, lung, and kidney tissues following initial burn wound infection by this bacterium. Consequently, it has been suggested that cyanide production by this bacterium may be closely related to the high mortality of patients suffering massive septicemia from burn infections (14, 44).

We are interested in the molecular structure and function of the CIO from *P. aeruginosa* and the significance of the CIO-terminated respiratory pathway in the physiology and ecology of *P. aeruginosa*. Therefore, in this present paper, we describe the isolation and characterization of a mutant strain of *P. aeruginosa* lacking CIO activity and we describe the cloning of the complementing gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this work and their relevant characteristics are shown in Table 1. Bacteria were propagated aerobically at 30°C on Luria-Bertani (LB) plates and in LB broth (34). As required, antibiotics were added at the following concentrations: streptomycin (1 mg ml^{-1}), tetracycline (300 μg ml^{-1}), and carbenicillin (500 μg ml^{-1}).

Preparation of membranes. Membranes were prepared essentially as described previously (23). Cells were grown in LB broth until the late logarithmic phase of growth (optical density at 600 nm = 1.0). Cells were pelleted by centrifugation at 10,000 × *g* and washed twice in 0.05 M potassium phosphate buffer (KP_i), pH 7.0. The cells were suspended in 0.01 M KP_i containing 5 mM $MgCl_2$ and 0.01 mg of DNase ml^{-1}, and this cell suspension was disrupted twice by passage through a French pressure cell at 16,000 lb/in^2. Following centrifugation at 10,000 × *g* for 10 min to remove unbroken cells, the membrane fraction was pelleted by centrifugation at 68,000 × *g* for 60 min. This fraction was washed in buffer and recentrifuged as described above, and the washed membrane pellet was resuspended in 0.01 M KP_i–5 mM $MgCl_2$ at protein concentration of 15 to 30 mg ml^{-1} before use.

Protein determination. The protein content was determined by the method of Markwell et al. (21), with bovine serum albumin being used as the protein standard.

Measurement of O_2 uptake and cyanide titration. Samples (3 ml) of membranes resuspended in 33 mM KP_i (pH 7.0) at a protein concentration of approximately 0.13 mg ml^{-1} were transferred to the O_2-electrode vessel at 25°C. Substrate was added to a final concentration of 33 mM (malate and succinate) or 0.33 mM (NADH). The electrode was calibrated with O_2-saturated buffer (~240 nmol of O_2 ml^{-1}), and then the mixture was made anoxic with a few grains of sodium dithionite. The effect of KCN on O_2 uptake was investigated by adding suitable amounts of freshly prepared KCN solution approximately 5 min after the addition of substrate and determining the rates of O_2 uptake before and after the addition of the inhibitor.

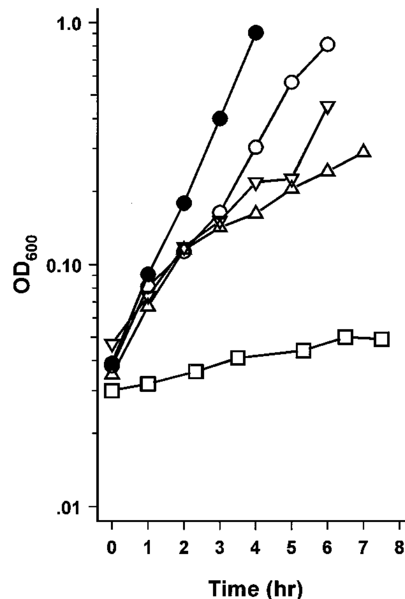

FIG. 1. Growth of various *P. aeruginosa* strains in the presence and absence of azide. Cultures were grown in LB broth in the presence (open symbols) and absence (closed symbols) of 1 mM sodium azide. ● and ○, PAO6049; □, PAO7701 *cio*; ▽, PAO7702 (*cio*/pLC1); △, PAO7703 (*cio*/pLC2). OD_{600}, optical density at 600 nm.

Analysis of cytochrome content of membranes. Analysis of the cytochrome content of membranes was carried out with a Shimadzu MPS-2000 dual-beam spectrophotometer. Washed membranes were resuspended in KP_i to a protein concentration of 2 mg ml^{-1}. Room temperature difference spectra (reduced-minus-oxidized and reduced-plus carbon monoxide-minus-reduced) were recorded essentially as described previously (18, 43). Samples were reduced with a few grains (≤5 mg) of sodium dithionite and oxidized in the presence of a small amount (≤5 mg) of ammonium persulfate. Carbon monoxide was introduced into reduced samples by gentle bubbling with CO for 3 min.

Cytochrome *c* oxidase activity. Cytochrome *c* oxidase activity was determined spectrophotometrically by monitoring the oxidation of exogenously added, reduced horse heart cytochrome *c* at 549 nm as previously described (27). The reaction mixture contained 40 μM dithionite-reduced cytochrome *c* in 50 mM KP_i, and the reaction was started by the addition of 100 μg of membrane protein. Results were calculated with an extinction coefficient for cytochrome *c* of 12.8 mM (27).

Mutagenesis and isolation of mutants. Insertion mutagenesis of the chromosome of *P. aeruginosa* PAO6049 was carried out with mini-D171 as described by Darzins et al. (8, 33). Phage lysate was produced by thermal induction of *P. aeruginosa* CD10 carrying the mini-D replicon pADD171. This lysate was used to transduce PAO6049 to tetracycline resistance at a multiplicity of infection of between 0.1 and 1.0. Tet^r transductants were pooled, and an azide-carbenicillin enrichment was carried out to enrich them for putative CIO mutants. The pooled transductants were inoculated into LB broth containing 1 mM azide and allowed to grow at 30°C for 60 min before the addition of carbenicillin (2 mg ml^{-1}). The rationale was that azide-resistant cells would grow and be lysed by the carbenicillin while azide-sensitive mutants would not be actively growing in the presence of 1 mM azide and so would survive the antibiotic treatment. Surviving cells were recovered by centrifugation, and the final survivors were plated onto LB-tetracycline plates. Tet^r survivors were then screened for their ability to grow on LB plates containing 1 mM azide.

In vivo cloning of complementing genes. *P. aeruginosa* chromosomal DNA capable of complementing the azide-sensitive growth phenotype of *cio* mutants was cloned with the in vivo system of Darzins et al. (8, 33). Briefly, a phage lysate of *P. aeruginosa* CD10 carrying the mini-D replicon pADD386 was prepared by thermal induction as previously described (8, 33). The lysate was used to infect

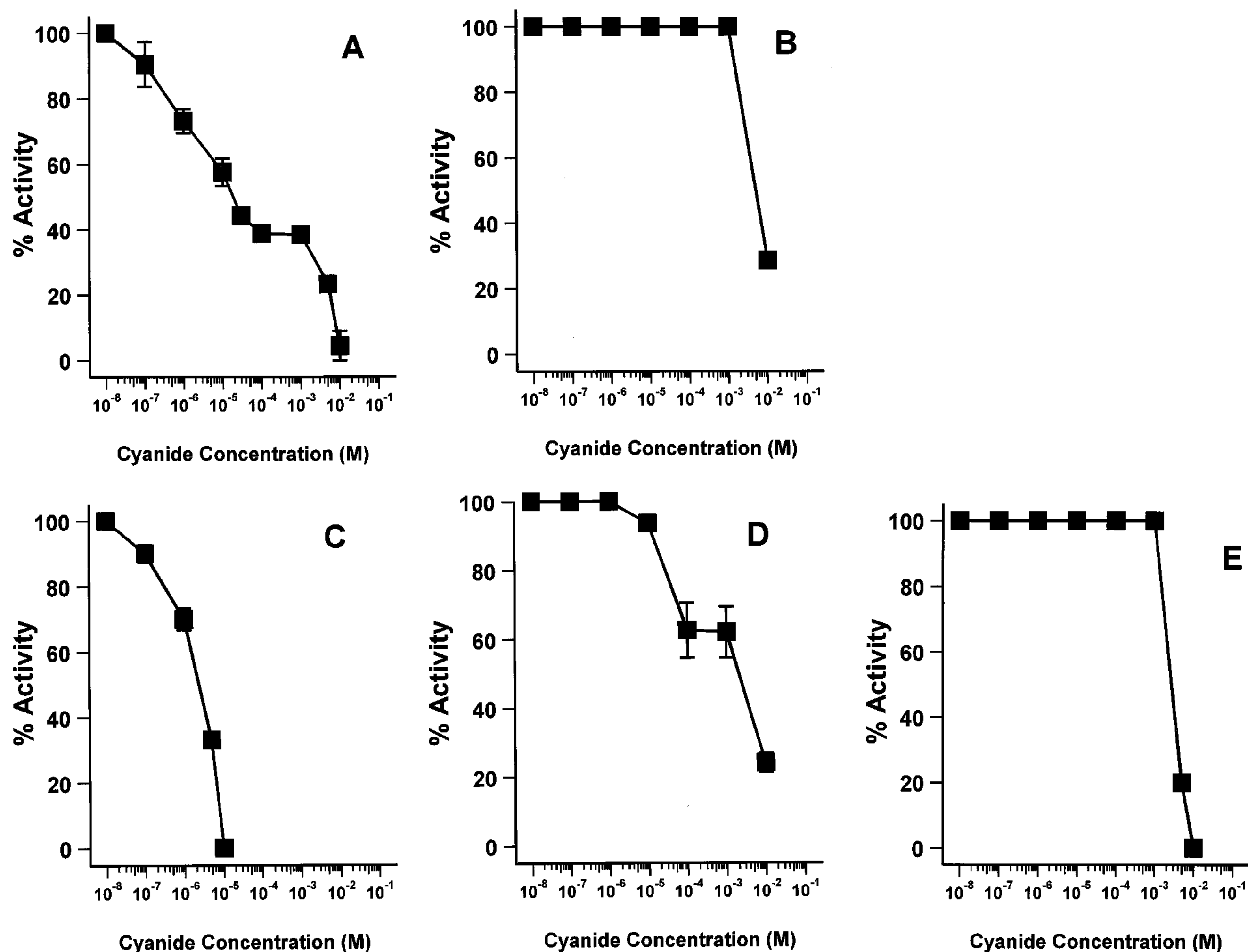

FIG. 2. The effect of KCN on NADH-dependent O_2 uptake of the membranes of *P. aeruginosa* strains. Activities were measured polarographically as described in Materials and Methods, with 0.33 mM NADH as the substrate. (A) PAO6049. (B) PAO6049 grown in the presence of 1 mM sodium azide. (C) PAO7701 *cio*. (D) PAO7702 (*cio*/pLC1). (E) PAO7703 (*cio*/pLC2). Error bars indicate the standard deviations of assays done in duplicate.

PAO7701, and the mixture was plated onto LB plates containing carbenicillin. Cb^r transductants were screened for their ability to grow on LB plates containing 1 mM azide. Subcloning of DNA from the initial pADD386-derived complementing clone was carried out by standard procedures (34) and by cloning into the *E. coli-P. aeruginosa* shuttle plasmid pUCP18 (37).

DNA methodology. Plasmid DNA was routinely prepared by the alkaline lysis procedure (34) and transformed into *E. coli* and *P. aeruginosa* as previously described (34). Standard methods for restriction enzyme digestion and ligation were used (34).

RESULTS

Rationale for mutant isolation. In order to isolate mutants defective in the CIO pathway, it was necessary to develop a reliable selection method for identifying mutants defective in cyanide-insensitive respiration. We reasoned that mutants defective in the CIO-terminated pathway would be more sensitive to cyanide inhibition of growth than would the wild-type strains. However, in our experiments, sodium azide rather than KCN was used because of its greater stability in an aqueous solution (16, 31). Preliminary experiments were carried out to determine the appropriate azide concentration for the selection of mutants defective in cyanide-insensitive respiration. Strain PAO6049 was grown in LB broth cultures and on agar plates in the presence of various concentrations of azide. As expected, increasing the azide concentration resulted in a slower growth rate in liquid culture. With 1 mM azide, the specific growth rate constant of the cultures was halved to 0.0075 h^{-1} (Fig. 1), but even in the presence of 5 mM azide, cultures grew reasonably well, and some very slow growth was still seen with 10 mM azide. On LB agar, colonies formed after 24 h with 1 mM azide, while with 2 mM azide, colony growth was only seen 36 h after inoculation. We grew cultures of *P. aeruginosa* PAO6049 in the presence and absence of 1 mM azide, prepared membranes from both cultures, and investigated the effect of cyanide on their NADH-dependent O_2 uptake. Figure 1 shows growth curves for PAO6049 grown with and without 1 mM azide. Figure 2A shows the expected biphasic titration curve for membranes from cells grown in the absence of azide. The titers indicate the presence of at least two terminal oxidases with differing sensitivities to cyanide, with the more cyanide-sensitive respiration being due to cytochrome *co* and the more resistant respiration being due to the CIO. In contrast, cells grown in the presence of 1 mM azide show essentially monophasic curves (Fig. 2B) corresponding to a terminal oxidase with high resistance to cyanide. The actual O_2 uptake rates were significantly higher for the azide-grown cell membranes, and this is exclusively due to an increase in the

TABLE 2. Comparison of the respiratory activities of various *P. aeruginosa* strains

Strain	NADH-dependent O_2 uptake (nmol of O_2 min^{-1} mg of $protein^{-1}$)	CIO activity (nmol of O_2 min^{-1} mg of $protein^{-1}$)[a]	% CIO activity[b]	Succinate-dependent O_2 uptake (nmol of O_2 min^{-1} mg of $protein^{-1}$)	Malate-dependent O_2 uptake (nmol of O_2 min^{-1} mg of $protein^{-1}$)	Cytochrome *c* oxidase activity (μmol of cytochrome *c* oxidized min^{-1} mg of $protein^{-1}$)
PAO6049 without azide	44	17	38.6	ND[c]	ND	22.6
PAO6049 with 1 mM azide	125	125	100	ND	ND	28.1
PAO6049[d]	70	27	38.5	48.8	52.5	52
PAO7701 *cio*[d]	32	0	0	24.2	23.6	40
PAO7702 *cio*/pLC1[d]	69	43	62.5	36	30	39
PAO7703 *cio*/pLC2[d]	110	110	100	44	46	17.7

[a] NADH-dependent O_2 uptake in the presence of 1 mM KCN.
[b] CIO activity as a percentage of the total NADH-dependent O_2 uptake activity.
[c] ND, not determined.
[d] This PAO6049 culture was grown as part of the same experiment as those of the strains indicated by the same superscript.

activity of the cyanide-insensitive pathway (Table 2). We found a sevenfold increase in cyanide-insensitive respiration in azide-grown cells (determined as respiration in the presence of 1 mM KCN), while the cytochrome *c* oxidase activity (which represents only the activity of the cyanide-sensitive cytochrome *co*) of cells grown with azide was essentially the same as that for cells grown without (Table 2). On the basis of these results, we decided to use 1 mM sodium azide to attempt to differentiate CIO-deficient mutants from wild-type strains following insertional mutagenesis.

Mutant isolation. Mini-D171, Tet^r insertion mutants of *P. aeruginosa* PAO6049 were generated as described in Materials and Methods. Approximately 2,000 Tet^r transductants were screened for their ability to grow on LB agar containing 1 mM sodium azide. Three transductants which were unable to grow on this medium and which retained their azide-sensitive phenotype upon purification and retesting were found. The further characterization of one of these mutants, PAO7701, is described in the remainder of this paper. The ability of the mutant to grow in LB broth in the presence of 1 mM azide was compared with that of PAO6049. In the absence of azide, growth of the mutant was similar to that of PAO6049 (data not shown), but PAO7701 did not show any appreciable growth in the presence of azide (Fig. 1).

Cytoplasmic membranes were prepared from PAO6049 and PAO7701, and cyanide titration of their NADH-dependent O_2 uptake was performed. The results are shown in Fig. 2. Membranes from the putative CIO mutant PAO7701 showed a striking difference from those of PAO6049. PAO7701 gave a monophasic titration curve, with a total abolition of O_2 uptake at 10 μM KCN (Fig. 2C). This clearly showed that membranes of PAO7701 had lost all CIO-dependent respiration. The mutation in PAO7701 will subsequently be referred to as *cio*, and it indicates a mutant defective in the CIO-terminated respiratory pathway. The O_2 uptake rates of the *cio* mutant were approximately 50% of wild-type levels (Table 2). We found a similar reduction in the levels of succinate- and malate-dependent O_2 uptake rates.

In vivo cloning of genes capable of complementing the *cio* mutation of strain PAO7701. We attempted to clone the wild-type *cio* allele with the bacteriophage D3112-based in vivo cloning system developed for *P. aeruginosa* (8, 33). Thermally induced mini-D386 lysates were used to transduce PAO7701 to the carbenicillin resistance carried by the mini-D element, and the transductants were screened for their ability to grow on LB agar containing 1 mM azide. A number of azide-resistant, Cb^r transductants were purified and then tested for their ability to grow in liquid cultures in the presence of 1 mM azide. Figure 1 shows that the growth rate of one such complemented mutant PAO7702 (PAO7701/pLC1) was restored to something close to that of the wild type. Membranes were prepared from eight such complemented mutants, and cyanide titration of the NADH-dependent O_2 uptake of one strain, PAO7702, is shown in Fig. 2D. The titers are biphasic and clearly similar to those of the Cio$^+$ strain PAO6049 (Fig. 2A). This result shows that CIO activity is restored by mini-D386-derived clone pLC1. A number of miniD-386 complementing clones were analyzed by restriction enzyme digestion, and they were found to contain insert fragments ranging from 15 to 30 kb; pLC1 contained a 15-kb insert.

A *Hin*dIII restriction digest of pLC1 was shotgun cloned into the broad-host-range plasmid vector pUCP18, a pUC18 derivative stabilized for use in *P. aeruginosa* (33). A pUCP18 recombinant clone, pLC2, containing a 5.2-kb *Hin*dIII fragment and complementing PAO7701 for growth on azide plates and in liquid medium was isolated (Fig. 1). The pLC2-complemented mutant, PAO7703, gave monophasic cyanide titers, but all of the respiratory activity was cyanide insensitive (Fig. 2E), indicating that the gene able to complement the defect in CIO activity was carried on the 5.2-kb insert.

Table 2 shows the actual values for the NADH-dependent O_2 uptake rates of PAO6049, the *cio* mutant PAO7701, and the mutants complemented with clones pLC1 and pLC2 (strains PAO7702 and PAO7703, respectively). The mutant PAO7701 had just 50% of the activity of the Cio$^+$ strain, but wild-type levels of activity were recovered in the pLC1-complemented mutant PAO7702. After the *cio* mutation was complemented by a multicopy replicon in PAO7703 (pLC2 is derived from pUCP18 which replicates to approximately 30 copies per cell), the oxidase levels for PAO7703 were more than 50% higher than those for the Cio$^+$ strain. More illuminating were the activities obtained in this strain for CIO-dependent O_2 uptake. This was determined as NADH-dependent O_2 uptake in the presence of 1 mM KCN, which, as the cyanide titers in Fig. 2A show, fully inhibits the cyanide-insensitive, cytochrome *co*-dependent respiration. As expected, PAO7701 had no activity, but the presence of the complementing plasmid pLC1 in strain PAO7702 resulted in a modest (~55%) increase in activity, while complementation by the multicopy replicon pLC2 in PAO7703 led to a more than fourfold increase in cyanide-insensitive respiration (Table 2). For a control, we also transformed PAO7701 with a pUCP18-derived plasmid (pLC3) carrying a noncomplementing fragment de-

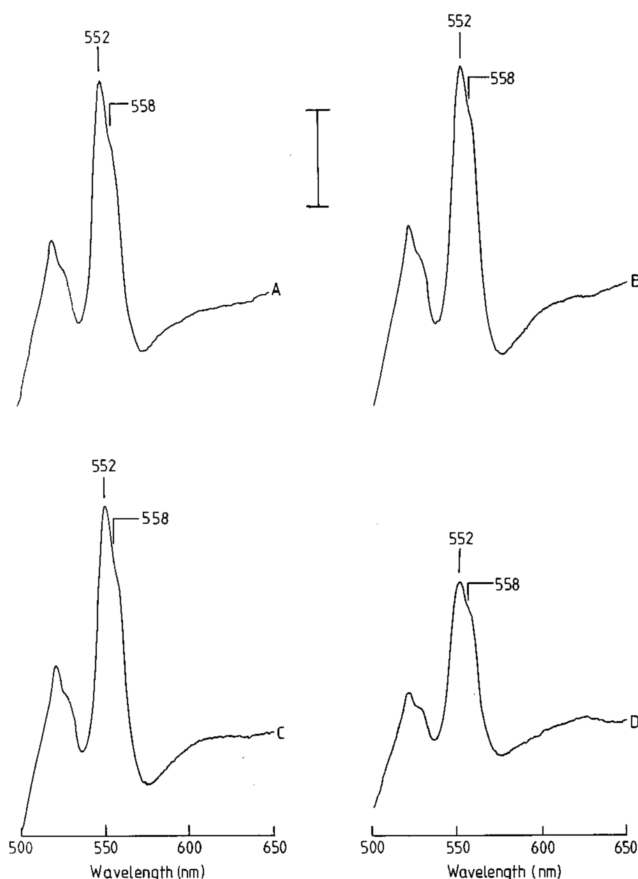

FIG. 3. Reduced-minus-oxidized difference spectra of membranes from various *P. aeruginosa* strains. Spectra were recorded as described in Materials and Methods. (A) PAO6049. (B) PAO7701 *cio*. (C) PAO7702 (*cio*/pLC1). (D) PAO7703 (*cio*/pLC2). The vertical bar represents a change in absorbance of 0.019, and all samples were at a protein concentration of 2 mg ml^{-1}.

rived from pLC2. This construction, PAO7704, did not show any increase in activity.

We also looked at succinate- and malate-dependent O_2 uptake rates, and the trends observed in the different strains were similar to those seen with NADH (Table 2). In PAO7701, the activities were about 50% of those of the Cio^+ strain, and wild-type levels were almost recovered in the complemented mutant. Table 2 also shows cytochrome *c* oxidase activities. The mutant strain PAO7701 has activities slightly lower than those of the wild type, but levels similar to those of PAO7701 are also present in the complemented mutant PAO7702. The cytochrome *c* oxidase activities of the wild type with the complementing multicopy plasmid pLC2 are further reduced. There were low levels in PAO7704 similar to those of the complemented wild type (see above, data not shown), suggesting that this result is a consequence of the presence of the multicopy replicon. These data indicate that the cytochrome *co*-terminated pathway was still functioning in the *cio* mutant.

Spectral analysis of the cytochrome composition of wild-type, *cio* mutant, and complemented-mutant membranes. Figure 3 shows the α and β regions of reduced-minus-oxidized difference spectra of washed membranes from PAO6049, PAO7701, PAO7702, and PAO7703 recorded at room temperature. The spectra were recorded from samples with identical protein concentrations (2 mg ml^{-1}) and are thus directly comparable. Strain PAO6049 shows an α maximum at ~552 nm due to *c*-type cytochromes and a prominent shoulder at 558 nm attributable to cytochrome *b* (Fig. 3A). Figure 3B shows a reduced-minus-oxidized spectrum of the *cio* mutant which is virtually indistinguishable from that of PAO6049, both qualitatively and quantitatively. The spectrum of membranes from a pLC1-complemented PAO7702 (Fig. 3C) is also virtually identical to that of the wild-type strain. The pLC2-complemented mutant, PAO7703 (Fig. 3D), gives a qualitatively similar spectrum, with signals at 552 and 558 nm present in the same proportions as in PAO6049 and the other strains. However, quantitatively, there is an approximately 30% reduction in the cytochrome levels. This is not simply due to the presence of the multicopy replicon, as the presence of the plasmid pLC3 (see above) does not reduce cytochrome levels (data not shown).

We obtained similar data from carbon monoxide difference spectra. The *cio* mutant PAO7701 showed no significant changes in CO binding between *b*- and *c*-type cytochromes, and the presence of the complementing plasmid pLC1 did not change the spectra signals (data not shown). In none of the spectra were signals observed which would have indicated the presence of cytochrome baa_3 in these strains of *P. aeruginosa.*

DISCUSSION

Recent work on the molecular genetics of bacterial terminal oxidases has led to the grouping of a majority of bacterial cytochrome *c* and quinol oxidases into a superfamily of heme-copper respiratory oxidases (5, 12). This grouping is based upon the overall homology of the amino acids and the similarities in the biochemical and biophysical properties of the heme prosthetic groups of these oxidases (5, 12, 13, 35). They closely resemble the catalytic subunits of eukaryotic mitochondrial cytochrome *c* oxidases (10). This contrasts somewhat with an earlier view that bacterial terminal oxidases were a diverse group of electron transfer proteins (29, 30). However, there are distinctive oxidases which do not fall into this superfamily, in particular the cytochrome *bd*-type oxidases of *E. coli* and *A. vinelandii* which show no homology with members of the oxidase superfamily (15, 26). We are interested in the terminal oxidases of the aerobic respiratory chain of the opportunistic human pathogen *P. aeruginosa* which include a cytochrome *c* oxidase, cytochrome *co*, and a cyanide-insensitive terminal oxidase of unknown molecular structure. We are investigating their structural and functional relationships to known oxidases and their role in the adaptation of *P. aeruginosa* to different environmental conditions. In order to facilitate these studies, we describe in this paper the isolation of a mutant of *P. aeruginosa* defective in the cyanide-insensitive respiratory pathway.

cio mutants were identified by their failure to grow on medium containing 1 mM sodium azide (a terminal oxidase inhibitor which acts in a similar manner as cyanide). The ability of this assay to distinguish such mutants was based on the assumption that in the presence of 1 mM azide, a fully functional CIO-terminated pathway would be required to permit aerobic growth of *P. aeruginosa*. The use of azide to differentiate between terminal oxidase mutants has been reported previously, e.g., to distinguish *E. coli* mutants defective in the cytochrome *bd* terminal oxidase (16, 31), although the concentrations of azide used in those studies were lower than those used in this study. In these cases, advantage was taken of the observation that the cytochrome *bd*-terminated branch of the aerobic respiratory chain of *E. coli* is less sensitive to azide and cyanide than is the alternative branch terminated by cytochrome *bo* (20). However, a potential problem with the use of azide as a screen is that, certainly in *E. coli*, other loci (*azi* locus [3]) not known to be related to respiratory metabolism might

be involved in the response to azide. Our preliminary studies indicated that the presence of azide actually stimulates the cyanide-insensitive respiratory activity of *P. aeruginosa* (Fig. 2A and B), potentially enhancing its use as a discriminating agent in the selection of *cio* mutants.

We show clearly that the isolated *cio* mutant has a phenotype deficient in cyanide-insensitive respiration. The mutant was isolated following insertion mutagenesis by its inability to grow on LB plates with 1 mM sodium azide. It was also severely affected in its ability to grow in liquid culture in the presence of azide (Fig. 1). However, as the mutant could still grow aerobically, it clearly had not lost a component essential to aerobic respiration and was presumably growing by making use of the cytochrome *co*-terminated pathway to O_2. The *cio* mutation had no observable effect on the spectroscopically detectable levels of cytochromes in membranes from aerobically grown cells, either in reduced-minus-oxidized (Fig. 3) or carbon monoxide difference spectra. A terminal oxidase cytochrome baa_3 has recently been purified from *P. aeruginosa*, and there have previously been reports of aa_3-type cytochromes in strains of this bacterium (44). We found no spectral evidence for cytochrome baa_3 in Cio^+ or Cio^- strains derived from PAO1 which were investigated as a part of this study. Membranes prepared from the *cio* mutant had lowered O_2 uptake rates with three different substrates (Table 2), but cytochrome *c* oxidase activity was reduced only modestly in the mutant (Table 2), supporting the spectral and cyanide titration data in suggesting that the cytochrome *c* oxidase, cytochrome *co*, is not affected by the *cio* mutation.

P. aeruginosa also synthesizes the oxygen-binding and -reducing heme protein cytochrome cd_1 (29, 45). This periplasmic enzyme, which reacts with NO_2^- as well as with O_2, was initially thought to be an oxidase, but its physiological function has clearly been identified as that of a nitrite reductase. The fact that cytochrome cd_1 is synthesized only in the presence of nitrate, is solubilized during cytoplasmic membrane preparation, contains a heme d_1 with very distinctive spectral characteristics, is cyanide sensitive, and oxidizes cytochrome *c* (29, 45) means that it obviously terminates a respiratory pathway quite different from the cyanide-insensitive respiration which is the concern of the present study.

Under the laboratory growth conditions used in this study (aerobic growth in LB medium), CIO appears to be redundant, as similar growth rates were found for Cio^+ and Cio^- strains in the absence of azide. It is possible that the presence of cyanide-insensitive respiration confers a growth and/or survival advantage only under cyanogenic growth conditions or under particular O_2 tensions. Certainly, in the presence of 1 mM azide, the Cio^+ strains have clear growth advantages over the *cio* mutant.

In our hands, the cyanide-insensitive respiration of *P. aeruginosa* is somewhat less resistant to cyanide than that reported by Matsushita and coworkers (25). They found that a 50% inhibition of NADH-dependent O_2 uptake was achieved by IC_{50}s of ~5 μM and 30 mM cyanide for cytochrome *co* and CIO, respectively. We typically found values of approximately 3 μM and 3 mM cyanide for the PAO1-derived strains used in this work. However, we also looked at strain IFO3445, the strain used by Matsushita et al. (23), and obtained IC_{50}s of 4 μM and 3 mM cyanide for cytochrome *co* and CIO, respectively (data not shown). The reason for the discrepancy with the earlier study is not clear, but what is certain is that we are dealing with a bacterium with an oxidase that is highly cyanide insensitive.

The precise nature of the lesion in the *cio* mutant is unknown at present, but there are a number of possibilities. The lesion could be in the structural gene for a polypeptide of CIO itself or in an essential electron transfer component unique to the cyanide-insensitive pathway. Alternatively, the mutation could lie in a gene required for the synthesis, insertion, or assembly of a prosthetic group associated uniquely with CIO. A further possibility is that a positive regulator of cyanide-insensitive respiration has been mutated. However, as the key phenotypic trait of the *cio* mutant is its total loss of cyanide-insensitive respiration and particularly as complementation of this phenotype in *trans* with the multicopy plasmid pLC2 leads to a greater than fourfold overexpression of cyanide-insensitive respiration (Table 2), we think it is probable that the *cio* mutation lies in a structural gene for CIO.

An intriguing question is the molecular nature of CIO. Limited circumstantial evidence which indicated that induction of CIO activity following the onset of stationary phase was not accompanied by any change in cytochrome composition led to the suggestion that CIO might be a non-heme oxidase (25, 40, 44). In this study, we found that while growth in the presence of 1 mM azide led to a sevenfold increase in cyanide-insensitive respiration, it was not accompanied by any changes in the composition of the membrane cytochromes (data not shown). The *cio* mutant did not appear to lose any cytochromes, and its complementation by pLC2, although resulting in a fourfold increase in CIO activity, did not lead to overexpression of any cytochromes; indeed, there was an unexpected drop in the overall levels of *b*- and *c*-type cytochromes. It is possible that this results from overexpression of the cloned *cio* gene product, but it certainly suggests that the gene product is not a heme protein. It is probable that CIO is a ubiquinol oxidase, as inhibitor titration experiments indicate that it branches from the main respiratory chain at the quinone-cytochrome *b* level and it oxidizes neither cytochrome *c* nor tetramethyl-*p*-phenylenediamine. Conclusive data from the purified oxidase are needed to establish this. There is no confirmed example of a non-heme terminal oxidase in bacteria. On the basis of inhibitor titers, Appleby suggested that nitrogen-fixing bacteroids of *B. japonicum* might contain a soluble, non-heme oxidase (2). However, all recent studies indicate that *B. japonicum* contains multiple terminal oxidases all related to the heme-copper superfamily of respiratory oxidases (12). The much studied but poorly defined cyanide-insensitive alternative oxidase of plant mitochondria has been partially purified by a number of groups and is reported to be a non-heme oxidase (4, 17, 19). However, it is characteristically inhibited by salicylhydroxamic acid, which has no effect on *P. aeruginosa* CIO activity (25). Therefore, the possibility that CIO of *P. aeruginosa* is a non-heme oxidase will be subject to further investigation, both via purification of the enzyme and sequence analysis of the cloned *cio* gene(s).

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